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(21) International Application Number: PCT/US90/04506 (22) International Filing Date: 16 August 1990 (16.08.90) (30) Priority data: 398,265 24 August 1989 (24.08.89) US (60) Parent Application or Grant (63) Related by Continuation US 398,265 (CIP) Filed on 24 August 1989 (24.08.89) (71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : LONG, George, Robinson [US/US]; 18 Deer Run, Newark, DE 19711 (US). LUOMA, Robert, Paul, II [US/US]; 406 Longfield Drive, Newark, DE 19713 (US). SAUNDERS, David, William [US/US]; 102 Wildwood Avenue, Pitman, NJ 08071 (US). STAVE, James, William [US/US]; 106 Fantasia Drive, Newark, DE 19713 (US). (74) Agents: FLOYD, Linda, A. et al.; E.I. du Pont de Nemours and Company, Legal Department, 1007 Market Street, Wilmington, DE 19898 (US). (81) Designated States: AT (European patent), AU, BE (European patent), BG, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), HU, IT (European patent), LU (European patent), NL (European patent), RO, SE (European patent), SU, US. Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: IMMUNOASSAY TO DETECT PSEUDOCERCOSPORELLA ANTIGEN IN CEREAL PLANTS (57) Abstract <p>A rapid, sensitive and specific immunometric assay system is provided for determining the presence and/or concentration of antigens associated with infection of cereal plants by the fungal pathogen <i>Pseudocercospora herpotrichoides</i> including a grinding apparatus for extracting such antigens in suitable liquid media. A polyclonal, monospecific antibody reagent is employed in a "forward-sandwich" immunoassay for detection of polyvalent fungal antigens associated with the development of cereal eyespot disease.</p>		

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TITLE

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IMMUNOASSAY TO DETECT
PSEUDOCERCOSPORELLA ANTIGEN IN CEREAL PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

10 This application is a continuation-in-part of
copending application Serial No. 07/398,265 filed
August 24, 1989.

Technical Field

15 This invention relates to a highly sensitive
and specific method for detecting polyvalent antigens
in plant tissue. In particular, it relates to a
rapid immunoassay for antigen associated with fungal
infection of wheat causing cereal eyespot disease.
20 Another aspect of this invention relates to a novel
grinding apparatus for extraction of antigens from
infected plant tissue.

Background of the Invention

25 Cereal eyespot (footrot) is a fungal disease of
wheat, rye, oats, barley, and grasses. The disease
is found in many parts of the world with cool, moist
climates and is especially prevalent in the
Great Lakes and Pacific Northwest regions of the
United States and in portions of South America,
30 Europe, New Zealand, Australia, and Africa. The
disease cycle begins in autumn or early spring when
Pseudocercospora herpotrichoides spores carried
over on field stubble from the previous year's crop
are deposited on the basal culm (foot) of the young
35 cereal plants by rain splash. The spores germinate
and superficially infect outer leaf sheaths. If

cool, moist conditions persist through the winter and
spring, the fungus penetrates into inner leaf sheaths
and eventually reaches the stem. The resulting
lesion disrupts the flow of nutrients to the upper
portions of the plant and may weaken the stem
sufficiently to cause the stem to be beaten down
easily or "lodged". The disease may kill the plant
outright, but more often results in reduced yields.
Yield reduction may result from poor head filling,
reduced seed numbers, or harvesting difficulties due
to lodging.

The presumptive diagnosis of cereal eyespot
disease primarily relies on the identification of
characteristic elliptical or "eye" shaped lesions.
Early in the season, lesions are found only on the
outer leaf sheaths and are very easily confused with
symptoms associated with other fungal cereal
diseases. Other foot diseases with similar symptoms
are "sharp eyespot" caused by Rhizoctonia solani
(cerealis), "take-all" caused by Gaeumannomyces
graminis, and "root rot" caused by Fusarium species.
Even late in the season when eyespot lesions are
fully developed, disease diagnosis is relatively
difficult. Confirmation of eyespot usually results
from a direct microscopic examination of the lesion
for the presence of P. herpotrichoides spores. This
technique is labor intensive and is not a reliable
method of confirming the disease.

Eyespot is the only early season foot disease
of wheat that usually warrants the application of
fungicide for control. While severe take-all and
sharp eyespot outbreaks may result in economically
important crop loss, the cost benefit ratio does not
usually justify treatment. Benzimidazoles and
certain sterol-biosynthesis-inhibiting fungicides

have been shown to be effective treatments for cereal
5 eyespot. Field data indicate that there is a two to
three week application window when fungicide
treatment results in maximum disease control. The
timing of this narrow application window depends on
weather conditions, cereal cultivar, and disease
10 pressure.

If eyespot is present in the field but symptoms
have not developed before the application window,
growers must make decisions to use fungicides without
knowing whether or not the disease is present. For
15 these reasons, a method to detect presymptomatically
the fungus associated with eyespot would result in
fewer, more effective fungicide treatments.

Many assays which diagnose disease and
quantitatively determine pathogen levels in animals
20 are based on immunological techniques. These methods
rely on the highly specific interaction of antibody
molecules with substances, referred to as antigens,
used to elicit antibody production. When an antigen
is introduced into the body of a vertebrate animal,
25 the animal's immune system reacts by generating
antibodies which bind to the antigen at immunogenic
sites. Large antigen molecules such as proteins and
polysaccharides typically contain many different
immunogenic sites. Antibody molecules produced by a
30 single antibody-producing cell are identical and are
referred to as "monoclonal" antibodies. The antibody
response to most antigens is "polyclonal" in that
many different antibody molecules are produced in
response to each of the immunogenic sites recognized
35 by the host. The antibody response to a given
antigen is determined genetically and varies between
species of animals.

Immunoassays are particularly well suited for
5 detection of polyvalent antigens that can complex
with two or more antibodies at the same time. One
such assay is referred to as a "sandwich" assay since
the antigen has two antibodies bound to its surface
at different sites. Unlabelled antibody (capture
10 antibody) immobilized on the surface of an insoluble
solid support matrix captures the antigen contained
within a fluid medium. After suitable incubation,
the solid support matrix is washed to remove the
fluid sample, including any unreacted antigen, and is
15 then contacted with a solution containing a known
quantity of labelled second antibody. The second
antibody may be "labelled" with a variety of
substances including radioisotopes, fluorescent
chromophores, or enzymes. During subsequent
20 incubation the labelled second antibody reacts with
the antigen fixed to the solid support matrix and in
so doing also becomes fixed. The solid support
matrix is washed a second time to remove unreacted
second antibody. The presence of antigen within the
25 test sample is demonstrated by detection of the bound
label. An assay based on this technique for
detection of the antigen associated with serum
hepatitis is described in U.S. Pat. No. 3,867,517,
and is referred to as a "forward-sandwich"
30 immunoassay. Variations of the assay format exist
and include: (1) reaction of the antigen sample with
the labelled second antibody before incubation with
the immobilized unlabelled antibody (referred to as a
"reverse-sandwich" immunoassay), and (2) incubation
35 of antigen with labelled and unlabelled antibody at
the same time ("simultaneous-sandwich" immunoassay).

Application of immunoassay technology to the field of plant pathology has been demonstrated by the work of Clark and Adams [J. Gen. Virol., 34, 475-483 (1977)]. The authors describe the use of a forward-sandwich enzyme immunoassay method to detect viral antigen in plant tissue. Methods are described to extract viral antigens from plant materials, immunize animals, purify polyclonal antibody reagents, and assay for the presence of viral antigen. The assay was both sensitive and specific for detecting viral antigens; however, the method suffers from several deficiencies. For instance, the authors utilized a relatively complex sample extraction buffer, large sample volumes, high (30°), low (4°), and room temperature incubations, and required 18-30 hours to complete. Despite its weaknesses, this assay method has served as a model for workers trying to develop immunoassays for plant pathogenic fungi.

Bolik et al. [Z. Pflanzenkr. Pflanzenschutz, 94, 449-456 (1987)] have reported development of an immunoassay for detection of *P. herpotrichoides* in artificially inoculated wheat stems. Rabbits were immunized with suspensions of freeze-dried mycelia. The resulting polyclonal antibodies were used to develop an immunoassay according to the method of Clark and Adams (supra). The assay detected both wheat and rye isolates of *P. herpotrichoides* but cross-reacted with other genera of important wheat pathogens and exhibited nonspecific reactions. Bolik et al. suggest that monoclonal antibodies be used instead of polyclonal reagents to overcome these problems.

A second immunoassay for detection of *P. herpotrichoides* in wheat was reported by Unger and

Wolf [J. Phytopath., 122, 281-286 (1988)]. Rabbits were immunized with ground mycelia and purified polyclonal antibodies were used to detect fungal antigens in plant extracts directly adsorbed to the surface of polystyrene plates. A complicated assay was developed that required 22 hours to complete and used three different incubation temperatures (37°, 6°, and room temperature). The authors reported reactivity to nineteen Pseudocercospora isolates and no cross-reactivity to twelve other wheat pathogens. However, the inherent low sensitivity associated with this assay format limits the value of the results. It generally is believed that the low sensitivity of this format results from adsorption to the solid support of nonspecific substances in the sample other than the antigens of interest.

Efforts to develop immunological diagnostic assays for fungal plant pathogens were reviewed by Dewey [J. Phytopath., 122, 281-286 (1988)]. The author emphasizes that lack of progress in this area reflects the difficulty of raising specific antisera that are specific and suggests the use of monoclonal antibodies (Mab) as a solution in this regard. Dewey refers to a Mab-based immunoassay for detection of Pseudocercospora, but makes no specific claims concerning this assay.

At this time there exists a clear need for a sensitive and specific assay to determine the presence and/or concentration of P. herpotrichoides that is also inexpensive, rapid, simple to perform, and capable of processing large numbers of plant samples. Furthermore, a method is needed that detects P. herpotrichoides before eyespot symptoms appear, at a time when fungicide application is most effective. The method also should distinguish

5 P. herpotrichoides from other stem disease organisms whose symptoms can easily be mistaken for eyespot. Finally, there exists a need for a suitable grinding apparatus for the extraction of antigens from infected plants.

10 Summary of the Invention

10 Applicants' invention is in part a grinding device, comprising a cylindrical tube, one end of the tube being closed with a circular base. The base of the tube having on its interior face a plurality of
15 radiating ridges constituting a first grinding surface. The grinding device also includes a plunger having a first end and a second end, the first end being cylindrical and having radiating ridges complementary to the ridges of the first grinding
20 surface constituting a second grinding surface. The first end of the plunger is slidably insertable into the cylindrical tube and axially rotatable therein. The second end of the plunger has a means for rotating the plunger within the cylindrical tube so as to grind material positioned between the first and
25 second grinding surfaces. The space between the inner surface of the tube and the first end of the plunger is such that ground material escapes from the first and second grinding surfaces along the inner surface of the tube but unground material does not
30 escape from the first and second grinding surfaces. Applicants' invention also includes a method for assaying the fungus Pseudocercospora, in which a fungal antigen is extracted from plant tissue into an aqueous medium and a reaction system is formed on a
35 solid support by contacting the aqueous medium containing the fungal antigen with a number of components. These components include

- 5 (1) a high titer polyclonal capture antibody
 monospecific for the fungal antigen;
 (2) a polyclonal antibody that reacts to the
 antigen;
 (3) a reporter mechanism.

10 The components are combined in proportions such that
a signal from the reporter mechanism is related to
the presence of, or concentration of, fungal antigen
initially present in the aqueous medium. The method
also includes measuring the signal from the reaction
system and relating the signal to the presence or
15 concentration of fungal antigen initially present in
the aqueous medium. Applicants' invention further
includes a kit for assaying for the
Pseudocercospora antigen in cereal plants. The
kit includes the grinding device and the components
20 of the assay previously mentioned.

Brief Description of the Drawings

Figure 1 is a schematic diagram showing
components of the Pseudocercospora assay.

25 Figure 2a is a graph demonstrating the
relationship between disease severity and
Pseudocercospora immunoassay results using
infected wheat plants that were greenhouse grown.

30 Figure 2b is a graph demonstrating the
sensitivity of the Pseudocercospora immunoassay
using samples of wheat plants infected in the field
with P. herpotrichoides.

35 Figure 3 is a bar graph showing the
cross-reactivity of a forward sandwich enzyme
immunoassay performed on fungal extracts of Septoria,
Gaeumannomyces and Fusarium using cross-reactive,
polyclonal, rabbit-anti-P. herpotrichoides
immunoglobulin (Ig) as the capture antibody.

Figure 4 is a bar graph showing the results of
5 absorbed, monospecific rabbit-anti-Pseudocercospora
Ig used as capture antibody in a forward sandwich
enzyme immunoassay and tested for cross-reactivity
against fungal extracts of Septoria, Gaeumannomyces,
and Fusarium antigens.

10 Figure 5 displays data showing that the assay
is sensitive enough to detect picogram quantities of
the P. herpotrichoides antigen.

Figure 6 demonstrates the utility of the tissue
grinding apparatus used in conjunction with the
15 Pseudocercospora immunoassay.

Figure 7 shows an elevation view of a grinder
tube and lid.

Figure 8 shows the top of the grinder tube base
which forms the first grinding surface.

20 Figure 9 shows the bottom of the grinder tube
base.

Figure 10 shows an elevation view of the
plunger.

Figure 11 shows the top of the plunger
25 including means for manual or automatic rotation.

Figure 12 shows the bottom of the plunger which
forms the second grinding surface.

Figure 13 shows a vertical section of the
assembled parts of the grinding device.

30 Figure 14 shows a vertical section of a
preferred embodiment of the grinding device.

Detailed Description of the Invention

The present invention describes a rapid,
35 sensitive, and specific method for determining the
presence and/or concentration of polyvalent and/or
multivalent antigenic substances of the fungus
P. herpotrichoides in a sample. The method also

5 employs a novel grinding apparatus for extraction of the antigen from plant material.

10 In the context of this disclosure, terms shall be defined as follows. "Fungal antigen" refers to any fungal substance or group of fungal substances which are capable of eliciting an immune response when injected into animals. Fungal antigens are discussed extensively by Longbottom and Austwick in Immunochemistry [ed. by Weir et al., Ch. 7 (1982)]. The term "immunogenic" refers to the intrinsic capacity of an antigen to elicit the production of antibodies when injected into an animal host. The term "polyvalent" refers to an antigen molecule having different immunogenic sites in response to which antibody molecules are produced. The term "multivalent" refers to an antigen molecule having multiple occurrences of a single immunogenic site. Among such antigenic substances may be mentioned polysaccharides, proteins, nucleic acids, lipids, and any aggregate or regularly ordered structure comprised of multiple or repeating units or combinations of such substances (e.g., glycoproteins, lipopolysaccharides, and lipoproteins). Among the specific fungal antigens which may be assayed by the process of the present invention are components of spores, hyphae, or cytosol including structural components, toxins, enzymes, elicitors, or other pathogenic factors whether they are secreted, extracted, crude, or purified. "Antibody" relates to a blood serum protein of the globulin fraction formed in response to antigen. "Polyclonal antibody" refers to any preparation containing heterogeneous populations of antibody molecules produced by genetically unique, clonally distinct antibody producing cells, whether derived from the serum of an animal or prepared artificially by mixing together

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different monoclonal antibodies. "Monoclonal
antibody" refers to homogeneous preparations of
antibody molecules produced by a single population of
cloned antibody producing cells. "Reaction system"
refers to the assembled components of the immunoassay
described herein. "High titer" refers to the
capacity of an antiserum to be active at dilutions of
1: 50,000 or greater.

Figure 1 shows the components of the
immunoassay assembled on a solid support (SS). The
capture antibody (C.Ab) is a monospecific polyclonal
rabbit-anti-P. herpotrichoides antiserum (RAP) Ig
that has captured Pseudocercospora antigen (P.Ag)
previously extracted from plant material into a
buffer. A cross-reactive labelled antibody (L.Ab) is
also bound to the Pseudocercospora antigen (P.Ag)
and also serves as the attachment point for a
reporter mechanism. The avidin-enzyme conjugate
(A-E), biotin (B), and the enzyme specific substrate
(S) are collectively known as the reporter
mechanism. An avidin-enzyme conjugate (A-E) was
attached to the biotin of the labelled antibody
(L.Ab). A substrate (S) introduced into the reaction
system and specific for the enzyme (E) of the
avidin-enzyme conjugate (A-E) produced a colored
product (CP) which is measured by means of a
spectrophotometer. As discussed in greater detail
herein, the order in which the components are
assembled on the solid support (SS) depends on the
conditions under which the assay will be used and the
objectives of the assay.

Extraction of Fungal Antigens from Plant Tissue

5 As *P. herpotrichoides* invades plant tissue, antigens are produced by the fungus. These antigens must be extracted from fungal and plant matrices before they can be detected by the assay. A consistent extraction method is required if the assay is to be both sensitive and reproducible. Since the
10 assay may be performed with both young and old plant tissue, as well as with dried straw, an extraction procedure capable of macerating fibrous material is required.

15 The present invention describes several ways of extracting fungal antigens from all types of plant materials. The two major components of the extraction system are the extraction solvent and the extraction apparatus. The solvent may be virtually
20 any aqueous medium. Water alone suffices, however various additives may improve assay performance. The addition of pH buffering agents such as di- and mono-basic sodium phosphate or tris[hydroxymethyl]-aminomethane (Tris) help maintain neutral pH in the
25 sample and yield more consistent results. The outer surfaces of plant parts are often quite hydrophobic and do not easily wet with water. The addition of a surfactant or wetting agent, such as polyoxyethylene-sorbitan monolaurate (Tween 20), octyl phenoxy
30 polyethoxyethanol (Triton X-100), or sodium dodecyl sulfate aids in the extraction of fungal antigens. It is often desirable to limit microbial growth in the extracted plant material, particularly if the samples are to be stored for any length of time prior
35 to assaying. Including a microbial growth inhibitor such as sodium azide, chloroacetamide or sodium ethylmercurithiosalicylate (thimerosal) is generally sufficient. Preferably, the extraction buffer

consists of a Tris buffered saline (TBS) or phosphate buffered saline solution (PBS) with azide or chloroacetamide added as a preservative and Tween 20 added as a wetting agent.

Extraction of fungal antigens from plant parts may be assisted by means of mechanical maceration of the plant and fungal biomass in the presence of extraction buffer. Maceration of soft, decomposed, or very young tissue may be accomplished by any number of techniques, including using Potter or Dounce type glass homogenizers, crushing between two hard surfaces, or repeated freezing and thawing. Older plants and dried straw require more rigorous techniques, such as grinding in a mortar and pestle with or without the assistance of a grinding aid such as sand or powdered glass. These techniques are very labor intensive and do not lend themselves to use outside of the laboratory or to the processing of large numbers of samples. The present invention describes a tissue grinding apparatus specifically designed to enhance the extraction of fungal antigens from all types of plant materials.

Grinding between rough surfaces such as sand paper or knurled plastic partially macerates some fibrous materials but the technique is tedious, inefficient and does not permit simultaneous introduction of the plant material into an extraction buffer. For this reason a grinding apparatus (10) illustrated in Figures 7 through 14 was designed having three parts: a grinding tube (12), a one- or two-piece plunger (24), and a lid (22). The grinding tube (12) is closed at one end by a circular base (14), the interior surface of which is a first

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grinding surface (16) consisting of ridges (18)
5 radiating from the center of the base (14). The base
(14) also has a central peg (20) that protrudes from
the base (14) within the grinding tube (12) and along
the tube's longitudinal axis. The head (26) of the
plunger (24) provides a second grinding surface (28)
10 of radiating ridges (18) that fits into the grinding
tube (12) to contact the first grinding surface
(16). The plunger head (26) may be made as a
separate unit from the plunger handle (32) in which
case interlocking key (48) prevents the handle (32)
15 and plunger head (26) from rotating independently.
The second grinding surface (28) is interrupted at
its center by a cavity (30) which fits over the
grinding tube's peg (20) along the longitudinal
axis. Thus positioned, the plunger (24) is free to
20 rotate within the grinding tube (12) and extends out
from the top of the grinding tube (12) in a handle
(32) having features such as grips (34) or a
depression (38) to assist either hand (34) or machine
(38) rotation. The design of the tube (12) and
25 plunger (24) is such that unground tissue sample is
held between the grinding surfaces (16) and (28) in
grinding zone (40), while macerated tissue is flushed
away from the grinding zone (40) by the extraction
buffer into the separation zone (42). This
30 separation is achieved by providing a restricted
clearance space (44) between the inner wall of the
tube (12) and the grinding head (26). In the present
embodiment, the restricted clearance space (44) is
0.007 of an inch. An approximate range for the
35 restricted clearance space is 0.005 to 0.013 inches.

The design of the grinding surfaces is critical
to the consistent performance of this invention.
Most designs result in clogging of the grinding
surface with plant material, thus greatly reducing

grinding efficiency. The present invention has
5 grinding surfaces (16 and 28) which do not clog. The
grinding surfaces (16 and 28) have ridges (18) which
extend radially from the center of each grinding
surface. The ridges (18) on the base (14) of the
tube (12) and the plunger grinding head (26) are
10 complimentary. The two surfaces interlock and
disengage repeatedly when one or both of the two
grinding surfaces are rotated about the tube's
longitudinal axis. The constant engaging/disengaging
action causes the grinding surfaces to abrade and
15 crush the plant tissue at their interface. As
described above, previously ground tissue is swept
away from the grinding zone (40) by the extraction
buffer decreasing the possibility of clogging. The
peg (20) and cavity (30) in the center of the
20 apparatus (10) excludes plant material from the
center of the grinding surfaces (16 and 28) where
grinding is less efficient due to reduced rotational
motion and the diminished size of ridges (18). The
peg (20) and cavity (30) mechanism is collectively
25 known as a grind enhancing and alignment means (31).
In the present embodiment, the space labelled (46)
between the central peg (20) and the inner wall of
the cavity (30) is 0.005 inches. An approximate
range for this space is 0.003 to 0.010 inches. This
30 alignment space (46) is less than the space (44)
between the inner wall of the tube and the plunger
head. Thus, the peg and cavity work together to form
a grind enhancing and alignment means (31).

Figure 14 illustrates a preferred embodiment of
35 the grinding device. The one piece tool/handle in
this embodiment eliminates assembly of the tool and
enhances reliability and cleanability. In this
embodiment the grinding surfaces (16 and 28) are not

perpendicular to the longitudinal axis of the tube (12). Rather they are at an angle such that
5 extraction buffer and fungal antigens from the macerated plant material flow by gravity to the bottom of the grinding zone (40) and then into the separation zone (42) through the restricted clearance space (44). Further, the angled grinding surface
10 also permits shedding of extraction buffer as the tool is withdrawn from the cup, thus avoiding spillage and enhancing cleanability. The angle employed is not critical and can vary from 10° to 45° from the horizontal, preferably between 15° and 25°.

15 There are 3 or more ribs (49) spaced equally around the plunger (24). The ribs strengthen the plunger and provide a mixing action during grinding.

The plunger handle (32) in this embodiment has an expanded upper portion (47) that fits closely
20 within the tube (12) such that the plunger can freely rotate. The clearance between the expanded upper portion (47) and the tube (12) can correspond to the clearance space (44). This feature helps stabilize the plunger during grinding and prevents materials
25 from splashing out of the tube.

The expanded upper portion (47) can have a hole (48) for sample removal. If desired, the expanded upper portion can be fitted with a lid and serve as a
30 reservoir for the extraction buffer.

The grinder (10) may be constructed out of metal, ceramic, glass, or plastic materials. Since the preferred use of the grinder is as an inexpensive, disposable apparatus, injection molded
35 plastics such as polypropylene, polystyrene, polyethylene, or polycarbonate are preferred. The grinder may also be used to macerate other

materials such as seeds or soil in which case a harder material such as glass-filled plastic is required. The lid (22) in Figure 7 is used to maintain the cleanliness of the tube (12) and to protect the fungal antigen once extracted.

Fungal Antigens

Antigens derived from P. herpotrichoides may be isolated from the natural environment or cultured in vitro under optimal conditions to enhance the expression of immunogenic and, preferably, diagnostic antigens of the fungus while minimizing the amount of non-specific material in the antigenic extract obtained. Culture media may be liquid, solid, synthetic, defined, complex, enriched, or supplemented or combinations of these including tissues or substances derived from or comprised of cereal plants, preferably wheat. Other culture considerations include temperature, light, atmosphere, aeration, length of incubation, and other factors obvious to those skilled in the art.

Specific antigenic substances may be isolated from live or killed cells. Fungal cultures can be conveniently separated into solid and liquid fractions by either filtration or centrifugation. Culture filtrates or supernatants may contain metabolites or extracellular fungal antigens. Preparation of these molecules generally requires concentration and specific procedures such as dialysis or ultrafiltration to separate out media components. The solid fraction can be further processed by mechanical or physical disruption (e.g., drying and grinding, homogenizing, freezing and

thawing and sonicating), extraction with solvents or
5 detergents, or digestion with enzymes, acids, alkali
or other chemical means. The processed solid
fraction can be further divided into cell wall and
cytoplasmic (cytosol) fractions as a result of such
disruption. Specific substances can be further
10 purified by methods such as chromatography,
electrophoresis, precipitation, dialysis, filtration,
or combinations of these and other techniques obvious
to those skilled in the art. Thus, the antigen may
be a spore, hyphae, cytoplasmic fraction, toxin,
15 enzyme, exoantigen, elicitor, polysaccharide,
protein, nucleic acid, or any aggregate or regularly
ordered structure composed of multiple repeating
units or combinations of such structures or
substances.

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Antibodies to Fungal Antigens

Fungal antigens may be injected directly into
animals capable of making antibodies to that
substance. Alternatively, the antigens may require
25 further preparation to enhance their immunogenicity
prior to injection. Such procedures include mixing
antigens with an appropriate adjuvant or conjugating
antigens to a suitable carrier molecule. Factors
affecting the immune response to injected antigens
30 include the dose, route, timing, number of
injections, genetic constitution of the animal (e.g.,
species and strain) and physical nature of the
antigen (e.g., polysaccharide vs. protein). The
choice of these factors for the immunization protocol
35 depends upon the desired characteristics of the
resulting antibodies. Applicants' goal was to design
an immunization protocol to yield antibodies that

5 react with high specificity and sensitivity to
biologically relevant fungal antigens. In the case
of plant fungal diseases, relevant antigens may
include extracellular substances such as exoantigens
or enzymes, factors involved in the pathogenesis of
the disease such as elicitors, or components of the
10 invading hyphae.

The sensitivity of sandwich immunoassays is
dependent upon the binding affinity of the antibody
for the antigen and the overall avidity or strength
by which the antigen is bound to the solid phase.
15 Antigens may interact at multiple sites with antibody
immobilized on the solid phase. Thus, the strength
with which polyvalent or multivalent antigens are
bound increases exponentially as a function of the
number of valences involved in their attachment.
20 Similarly, polyclonal antibodies immobilized on the
solid phase may be expected to bind polyvalent
antigens with greater avidity than monoclonal
antibodies due to the inherent heterogeneity of the
polyclonal reagent. Greater sensitivity can be
25 achieved in forward sandwich immunoassays by
employing monoclonal antibodies having affinities for
their respective antigens of at least 10^8 M^{-1} and,
preferably, of 10^9 M^{-1} . [U.S. Pat. No. 4,486,530]
However, it is well documented in the scientific
30 literature that antibodies to certain antigens (e.g.,
polysaccharides) only have affinities ranging from
 10^4 M^{-1} to 10^6 M^{-1} . This is particularly evident in
the highly inbred strains of mice used for the
production of monoclonal antibodies. From these
35 considerations it may be expected that preparations
of polyclonal antibodies would provide more sensitive
immunoassays to detect such antigens.

Specificity of an antibody for an antigen is determined primarily by their complementary fit and the strength of the binding between the two molecules. With respect to specificity of binding, a preparation of monoclonal antibody is homogeneous whereas a polyclonal preparation is heterogeneous. Preparations of polyclonal antibodies can be made more specific by selectively removing those antibody molecules that cross-react. This can be done by immunoabsorption of such preparations with antigens to which the reagent cross-reacts. The specificity of polyclonal reagents can also be improved by selectively purifying only those antibody molecules specific for the antigen of interest by means of immunoaffinity chromatography. This method can be time consuming, result in low yields and loss of high affinity antibodies from the population. In contrast, immunoabsorption techniques remove cross-reactive antibodies in a one step process, do not deplete high affinity antibodies, and can result in high yields. As shown in the Examples, Applicants used immunoabsorption techniques to create a monospecific antibody reagent by removing cross-reactive antibodies.

The choice of animal species or strains in which to produce antibodies is a critical factor of the immunization protocol and directly effects the final performance of any immunoassay. The prerequisite step to developing an immunoassay is the production of a high titer antiserum. Antibody production to a given antigen is determined genetically and varies between species and between strains within a species. Presently, monoclonal antibodies are derived almost exclusively from mice.

As stated previously, the current art argues for the use of monoclonal (i.e., mouse) antibodies over polyclonal antibodies to increase both specificity and sensitivity of fungal immunoassays. However, mice, especially the highly inbred strains used for the production of monoclonal antibodies, are highly restricted in their capacity to make antibodies to many antigens, including polysaccharides which are the primary components of the hyphal cell wall. It has been demonstrated that mice produce low titer, low affinity antisera when injected with preparations of fungal antigens. Thus, it may not be possible to isolate monoclonal antibodies with the desired sensitivity and specificity to detect important diagnostic fungal antigens. In contrast, rabbits of the type used routinely for the production of antibody reagents are outbred animals which frequently exhibit strong antibody responses to antigens which mice respond poorly to, including fungal antigens. While the current art champions the potential of monoclonal antibodies to increase the specificity and sensitivity of fungal immunoassays it has failed to realize the limitations of this technology imposed on it by the genetics of the mouse immune system.

Considering the short time requirements and low cost associated with development of polyclonal reagents as well as the sensitivity and specificity of the resulting assay, polyclonal antibody reagents offer many advantages over monoclonal reagents.

High Titer Polyclonal Capture Antibody Monospecific for Pseudocercospora Antigen

The present invention uses an immunoabsorption technique to increase specificity by eliminating the

cross-reactivity of rabbit-anti-P. herpotrichoides (RAP) polyclonal antiserum to non-Pseudocercospora antigens. As used herein, the term "immunoglobulin" refers to antibody and "monospecific" refers to preparations of Ig that have been rendered specific for Pseudocercospora by immunoabsorption with cross-reacting fungi. There are a number of ways to prepare immunoabsorbed Ig. Available techniques include liquid phase immunoprecipitation with soluble fungal antigens, solid phase immunoabsorption with insoluble fungal components, and solid phase immunoabsorption with fungal antigen-solid support matrix conjugates. Each of these techniques involve treating the RAP Ig preparation with antigens prepared from cross-reacting fungi in order to specifically bind and remove cross-reactive antibodies. Fungi which may be used to immunoabsorb a RAP Ig reagent include Septoria tritici, S. nodorum, G. graminis, E. novali, E. culmorum, E. rosium, Rhizoctonia solani, Rhynchosporium secalis, P. anguioides, P. aestiva, as well as many other fungi which grow on cereal plants as pathogens or saprophites. The resulting monospecific Ig preparation will not react with fungal extracts to which it has been absorbed, yet retains reactivity towards P. herpotrichoides antigens.

30 Solid Support Matrix Immunoabsorbant

Covalently attaching an antigen, including its derivatives and analogs, to a solid support depends upon the particular molecular architecture of both types of molecules. Antigens contain functional groups such as amines, amides, carboxyls, 35 sulfhydryls, hydroxyls, and/or aldehydes to which a

5 support matrix with its own appropriate functional groups could be attached directly or indirectly. The attachment chemistry may vary depending upon the functional groups involved and the desirability of including a spacer arm between the antigen and the solid support. The resulting matrix must retain the ability to react with antibody.

10 In the case of fungal antigen-agarose matrices, a number of coupling chemistries are available. When hydroxyl groups are present upon two adjacent carbon atoms within the structure of carbohydrate molecules, they can be treated with sodium periodate to form two aldehyde groups. These groups can attach directly to amino-functional agarose through a Schiff's base intermediate which can be reduced to a stable carbon-nitrogen bond. Such reduction can be carried out by conventional means such as either sodium borohydride or sodium cyanoborohydride. This sequence of reactions, commonly referred to as "periodate oxidation and borohydride reduction", results in an antigen column capable of binding antibody.

25 Alternatively, carbohydrate could be attached to agarose through a spacer arm, such as 1,6-diaminohexane, diaminodipropylamine, or other bifunctional crosslinking agents after periodate oxidation. The use of a spacer arm can enhance the immunochemical reactivity of the conjugate. A number of crosslinking agents, both homo- and hetero-bifunctional, have been described in the literature and are available commercially [Pierce Bio-Research Products Technical Bulletin, "Double-Agents", Bifunctional Crosslinking Reagents, Vol.3 Pierce Chemical Co., Rockford, IL, U.S.A., 1982]. These

5 crosslinking agents can be used in conjunction with many different linkage strategies, depending on which functional groups are available for conjugation on both the antigen and solid support.

10 Carbodiimide crosslinking agents, such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, can also be employed to link antigens with carboxyl groups to amino groups on amino-activated agarose or conversely, to link the amino groups of the antigen to carboxyl groups on carboxyl-activated agarose.

15 Alternatively, support matrices may be purchased from commercial suppliers which have been pre-treated with crosslinking reagents. These "activated" supports will react directly with antigen functional groups when mixed in the appropriate coupling buffers. Examples of activated agarose support matrices and the functional groups to which they react are listed in Table 1.

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TABLE 1

5	MATRIX	
	<u>IMMOBILIZED</u>	<u>LIGAND</u>
10	Cyanogen Bromide- Activated Agarose	Amines, Proteins, Nucleic Acids, Polysaccharides
15	Epoxy-Activated - Agarose	Amines, Hydroxyls, Polysaccharides, Thiols
	Nitrophenyl Chloroformate - Agarose	Amines, Proteins
20	Aminohexanoic Hydroxy Succinimide - Agarose	Amines, Proteins
25	Thiol - Agarose	Thiols, Alkyl & Aryl Halides, C=O, C=C, C=N
	Carbonyldiimidazole - Agarose	Amines, Proteins
30	Carboxymethyl Cellulose Hydrazide and Polyacrylamide Hydrazide	Amines, Proteins, Polysaccharides

35

5 The chemical reactions involved in binding antigen molecules, or their derivatives or analogs, to the solid support are known to those skilled in the art. The important features of a particular antigen-support matrix are (1) that it react with an antibody specific for fungal antigen, and (2) that the matrix be stable under conditions of storage and use, i.e., that it not release antigen. While covalent bonds have been formed to ensure stability, bonding can involve other types of interactions which result in stable combinations of antigen and support, such as ionic or hydrophobic bonds. Such bonding methods are within the scope of this invention. Additionally, the molecular structure of the antigen derivative actually incorporated onto the solid support can vary depending upon the particular bonding reactions used and the optional use of a spacer arm. Such molecular structural variations to achieve bonding are not critical to practice the invention. Thus, "matrix" means any antigen molecule attached, directly or indirectly, to a solid support.

25

Pseudocercospora Immunoassay

The assay to detect *P. herpotrichoides* antigen in infected plant tissue may be configured in numerous formats. All formats require immobilization of antigen or capture antibody onto a solid support. 30 The solid support matrices may be microtiter plates, tubes, beads, magnetic particles, membranes and other well known supports.

In the case of immobilized antigen, the plant extract is adsorbed directly to the solid support and then reacted with a labelled antibody specific for fungal antigen. Since both plant material and fungal 35

antigen adsorb to the support, the fungal antigen density on the solid support is limited by the presence of non-specific plant material. This usually results in low assay sensitivity. It is important to detect the presence of P. herpotrichoides in wheat stems before the expression of symptoms. Therefore, it is desirable to have an assay with high sensitivity. The use of immobilized capture antibody in a forward-sandwich assay as schematically shown in Figure 1 is understood to be more sensitive than reverse or simultaneous sandwich assays. The capture antibody (C.Ab) is adsorbed to the solid support (SS) and then reacted with plant extract. The capture antibody (C.Ab) serves to selectively immobilize the fungal antigen (P.Ag) from the plant extract and results in a high specific antigen density on the solid support. The bound fungal antigen (P.Ag) is then reacted with a labelled second antibody (L.Ab).

Several grades of immunoreagent may be used to perform the assay. Generally, purified Ig reagents improve assay performance. Assay specificity is achieved by the use of immunoabsorbed reagents. The capture antibody, second antibody, or both, may be immunoabsorbed. Using the capture antibody as the only immunoabsorbed reagent may result in slightly improved assay sensitivity over other configurations.

Reporter Mechanism

The choice of reporter mechanism used to indicate the presence of bound antigen includes radioisotopes, fluorescent chromophores, chromophoric particles and enzymes. Enzymes are preferred due to the availability of sensitive chromogenic substrates

and the availability of simple instrumentation to
5 quantitate results. The use of horseradish
peroxidase may be limited due to the presence of
endogenous peroxidase activity in plant and fungal
extracts which can result in high non-specific color
development. Alkaline phosphatase is the preferred
10 label and its use results in low nonspecific color
development and excellent assay sensitivity. For use
in microtiter plate assays, the applicants used the
soluble yellow color signal produced by the enzyme
substrate p-nitrophenyl phosphate and measured the
15 signal as described in Examples 1g, 1h, 1i and 2.
With other assay formats, 5-bromo-4-chloro-3-indolyl
phosphate, p-toluidine salt may be used in conjunction
with a tetrazolium salt, such as nitroblue
tetrazolium chloride, to yield an insoluble formazan
20 dye precipitate. In either case, the color signal is
related to the presence or concentration of fungal
antigen initially present in the aqueous medium.

The reporter mechanism may be prepared using
several techniques. The first technique requires the
25 use of chemical conjugation methods similar to those
described in the "Antigen Support Matrix
Immunoabsorbant" section above. The result is an
immunoglobulin molecule covalently coupled to one or
more label molecules. Alternatively, immunoglobulin
and the label may be coupled via non-covalent
30 techniques, such as with the use of a
streptavidin-biotin system or the avidin-biotin
system as taught in U.S. Patent No. 4,228,237.

35 Field Testing Kit

Applicants' kit format for their invention
permits convenient, fast testing in the field of

5 cereal crops suspected of being infected with the
Pseudocercospora fungus. The kit will include the
grinding device and the preparations of the various
assay components described herein.

EXAMPLES

10 Unless specified otherwise, in all statements
of assay conditions and the preparation of reagents
the following are meant: temperature is in °C;
concentrations referred to as percentages are by
weight/volume; and abbreviations utilized are: ml
15 (milliliter), ul (microliter), M (molar), mM
(millimolar), g (gravity), MW (molecular weight), rpm
(revolutions per minute), nm (nanometer), um
(micrometer), h (hour), min (minute).

EXAMPLE 1a

Preparation of Fungal Extracts

20 Separate culture extracts were prepared from
five species of pathogenic wheat fungi. The extracts
served as both individual antigen sources for
immunoassay testing and as a source of antigen for
25 the immunoabsorption procedure described in Example
1f.

Culture flasks containing 100 ml of sterile
Potato Dextrose Broth (Difco, Detroit, MI) were
30 inoculated with the spores and hyphae from one of the
following fungi: P. herpotrichoides, S. tritici,
E. roseum, R. solani, or G. graminis. Cultures were
shaken at 100 rpm for one to two weeks at 23°.
Following the incubation period, hyphae and culture
35 supernatants were separated by centrifugation at
3000xg for 10 min and stored frozen at -20°.

Fungal antigens were then prepared by sodium
cholate extraction of the spent culture media and

5 macerated hyphae. Culture medium was added to hyphal pellets to result in a hyphal suspension of approximately 1 ml of media per 1 ml of packed hyphae. The hyphal suspensions were adjusted to 0.1% sodium cholate by the addition of a 1% stock solution in PBS (20 mM sodium phosphate, 140 mM sodium chloride) containing 0.05% sodium azide (PBS/azide).
10 The hyphae were thoroughly homogenized in Dounce-type tissue grinders. Homogenates were centrifuged at 3000xg for 10 min at 4° and the resulting cell pellets were discarded. The supernatant fractions were combined with the remainder of the spent culture media and the mixtures were dialyzed exhaustively
15 against two changes of saline (140 mM sodium chloride) followed by a final buffer change into coupling buffer (100 mM sodium bicarbonate, 500 mM sodium chloride, pH 8.5) using dialysis membranes with an 8,000 MW cutoff. The dialyzed extracts were concentrated to approximately 10 ml by
20 ultra-filtration in stirred-cells (Amicon, Bedford, MA) using 10,000 MW cut-off membrane filters.

25

EXAMPLE 1b

Extraction of Fungal Antigens from Plant Tissue

Both greenhouse- and field-grown wheat plants were tested separately in the assay. Antigen sources included fresh, frozen and dried plants. Plants were
30 prepared for extraction by cutting one to two inch stem segments measured up the plant from the basal culm to just above the coleoptile. Stems were extracted in PBS containing 0.05% Tween 20 (PBS/Tween) using a mortar and pestle. The resulting
35 plant extracts were centrifuged at 3000xg for 1 min to remove large debris and stored at 4° until

assayed. The extract derived from the ten stem
segment in 1 ml of extraction buffer is defined as
5 "undilute". In the present example, one
field-collected stem segment extracted into 5 ml of
buffer is defined as a 1:50 starting dilution and one
green house collected stem segment extracted into 10
10 ml of buffer is defined as a 1:100 starting dilution.

EXAMPLE 1c

Preparation of Immunogens and Rabbit Immunization Protocol

15 A first immunogen was prepared by culturing
four isolates of *P. herpotrichoides*, including wheat,
rye and Pacific Northwest strains in Potato Dextrose
Broth (Difco). The cultures were homogenized and
fungal fragments counted using a microscope and
hemacytometer. All four strains were diluted in PBS
20 and pooled to yield a mixture containing
approximately equal numbers of fragments of each of
the four strains. The pooled mixture (4×10^5 fungal
fragments per ml) was emulsified with an equal volume
of either complete Freund's adjuvant (CFA) or
25 incomplete Freund's adjuvant (IFA)
prior to injection into the animals. Three
New Zealand white rabbits were given two 1 ml
injections of the fungal-adjuvant emulsion
subcutaneously (SC) at multiple sites on days 0 (CFA)
30 and 24 (IFA).

A second antigen was prepared by grinding into
powder the lyophilized hyphae of *P. herpotrichoides*
(a wheat strain) grown in mineral dextrose yeast
(MDY) broth. The powdered hyphae (1 gram) was
35 resuspended in 10 ml PBS and centrifuged to pellet
insoluble material. The clarified supernatant
(optical density at 280 nm = 4.9) was sterilized by

filtration through a 0.45 um membrane (Gelman, Ann Arbor, MI) and used to immunize previously injected New Zealand white rabbits. Each rabbit was injected with 0.5 ml antigen B intravenously (IV) and 0.5 ml emulsified with 0.5 ml IFA intramuscularly (IM) on day 81. Antiserum was harvested from rabbits bled on days 0, 39, 49, 88, 91 and 95. A third immunogen (C) was prepared from a wheat strain fungal extract (Example 1a). The concentrated extract was centrifuged at 7800xg for 20 min and the supernatant sterile filtered. The sterile supernatant was emulsified with an equal volume of IFA and injected on days 140 and 168 (0.5 ml IM and 1.0 ml SC). Antisera were harvested from rabbits bled on days 154, 182 and 187.

EXAMPLE 1d

Purification of Rabbit-anti-
P. herpotrichoides Immunoglobulin (RAP Ig)
Purified RAP Ig was prepared from pooled antisera harvested from two of the rabbits bled on days 182 and 187. Fifty-five ml of antisera were filter sterilized, mixed with an equal volume of saturated ammonium sulfate and incubated 1 h at 4°. The precipitated Ig was harvested by centrifugation, dissolved in a small volume of saline and, dialyzed in 14,000 MW cutoff dialysis tubing 48 h in two changes of PBS at 4° (2 liters each change). The dialyzed Ig solution was centrifuged at 3000xg for 20 min to remove precipitated material. A Protein A Sepharose 4B slurry (32 ml, Pharmacia, Piscataway, NJ) was poured into a column (2.5 x 20 cm) and equilibrated with 300 ml 3 M NaCl, 1.0 M Glycine, pH 8.8 (U.S. Patent No. 4,704,366). The clarified Ig solution was

5 mixed with an equal volume of equilibration buffer
and allowed to pass through the column at a rate
determined by gravity (approx. 1 ml/min). The column
was washed with equilibration buffer until the
absorbance at 280 nm reached the baseline. Antibody
was eluted from the column with 53 ml of 0.1 M sodium
citrate, pH 3.0 and dialyzed overnight at 4° in two
10 changes of PBS (4 liters each change).

The concentration of purified Ig was determined
using an extinction coefficient $E(1\%, 280 \text{ nm})=14.3$.

EXAMPLE 1e

15 Preparation of Biotinylated Rabbit-anti-
P. herpotrichoides Immunoglobulin Conjugate
Purified RAP Ig (100 mg, 5 mg/ml PBS) was
diluted with an equal volume of 0.1 M bicarbonate
buffer pH 8.1. The diluted Ig was dialyzed with
20 14,000 MW cutoff dialysis tubing for 24 h at 4° in
two changes of 6 liters 0.1 M bicarbonate buffer.
N-Hydroxy-succinimidobiotin (Pierce, Rockford, IL)
was dissolved in dimethyl sulfoxide (5 mg/ml) and 4.2
ml reacted with the dialyzed Ig (40 ml) for 4 h at
25 room temperature. The resulting conjugate solution
was dialyzed against two changes of PBS (6 liters
each change) for 96 h at 4°. The biotin-Ig conjugate
was prepared for storage at 4° by addition of bovine
serum albumin (1% final concentration), glycerol (10%
30 v/v), sodium azide (0.1%) and sterile filtration.

EXAMPLE 1f

Immunoabsorption of Cross-reactive Rabbit-Anti-
35 P. herpotrichoides Immunoglobulin (RAP Ig)
The following protocol illustrates that
purified RAP Ig cross-reacts with antigens prepared

from numerous other plant pathogenic fungi when
tested using a forward-sandwich enzyme immunoassay
(Figure 3).

The cross-reactive Ig was removed by a solid
phase immunoabsorption technique to yield
monospecific RAP Ig. Fungal extracts from Example 1a
of S. tritici, F. roseum, R. cerealis, and G.
graminis were coupled separately to cyanogen
bromide-activated Sepharose 4B (Pharmacia AB,
Laboratory Separation Division, Uppsala, Sweden).
For each preparation, one gram of dried gel was
suspended in 33 ml of 1 mM hydrochloric acid (HCl)
and transferred to a Buchner funnel. The gel was
washed continuously with a total of 300 ml of HCl
over 15 minutes. After the acid wash, the gel was
rapidly filtered down to a moist paste, transferred
to a polypropylene centrifuge tube, resuspended into
1 gel bed volume of coupling buffer, and centrifuged
at 500 x g for two minutes. The gel pellet was saved
and immediately combined with approximately 10 ml of
fungal extract. The antigens were allowed to react
with the activated gel matrix for 2.5 h at room
temperature for Septoria, Fusarium, and
Gaeumannomyces or overnight at 4° for Rhizoctonia,
with gentle mixing under both conditions. Following
incubation, the gel was washed twice with 1 ml of
coupling buffer. Remaining reactive groups on the
Septoria, Fusarium, and Gaeumannomyces gels were
blocked using 1 M ethanolamine, pH 8.0, for 2 h at
room temperature. Reactive groups on the Rhizoctonia
gel were blocked using 10 mM Tris, pH 7.4, 140 mM
sodium chloride, 0.05% sodium azide (TBS/azide)
incubated overnight at 4°. The fungal
antigen-Sepharose 4B immunoabsorbants were packed

into 1.0 x 10 cm chromatography columns and
5 sequentially washed with 0.1 M sodium acetate, 1 M
sodium chloride, pH 4.0, and 0.1 M sodium carbonate,
1 M sodium chloride, pH 9.0, and 3 M sodium
thiocyanate in PBS followed by PBS/azide prior to
use. Final column bed volumes were 3-5 ml.

10 Purified RAP Ig at 5.2 mg/ml in PBS was
sequentially passed through the G. graminis,
S. tritici, F. roseum, and R. solani immunoabsorbant
columns. The Ig preparation was allowed to pass
through the columns at approximately 1 ml/min. The
15 columns were washed with PBS/azide until the
absorbance of the column effluents at 280 nm returned
to zero. Each pass through an affinity column
resulted in a 2-3 fold dilution of the RAP Ig which
was concentrated back down to the approximate
20 starting volume in a stirred-cell using a 100,000 MW
cut-off membrane filter.

Figure 4 is a bar graph showing the results of
absorbed monospecific RAP Ig used as capture antibody
in a forward sandwich enzyme immunoassay and
25 demonstrating cross-reactivity to fungal extracts of
Septoria, Fusarium, and Gaeumannomyces antigens.

EXAMPLE 1g

Pseudocercospora Immunoassay

30 The Pseudocercospora Immunoassay on plant
tissue suspected of being infected with
P. herpotrichoides was performed. The format
consisted of a 96 well microtiter plate. The general
assay protocol, consisted of 50 ul reagent additions
35 per well, 1 h reagent incubations at 25° followed by
four washes with 10 mM Tris, pH 7.4, 140 mM sodium
chloride, 0.05% sodium azide, 0.05% Tween 20
(TBS/Tween). RAP Ig (cross-reactive or monospecific)

was diluted to approximately 10 ug/ml in 10 mM bicarbonate coating buffer at pH 9.6 and added to microtiter plate wells. Samples were diluted in TBS/Tween and incubated with antibody coated wells. After the plates were incubated and washed, biotin-conjugated RAP Ig diluted to approximately 10 ug/ml in TBS/Tween was applied to the wells. Final reagent additions consisted of avidin-alkaline phosphatase conjugate (Organon Technica, Malvern, PA) diluted 1:250 into TBS/Tween followed by phosphatase substrate solution (1 mg/ml p-nitrophenyl phosphate, 2.0 mM magnesium chloride, 0.2 mM zinc acetate in 1.0 M diethanolamine buffer, pH 9.8). Color development in the wells was measured spectrophotometrically at 405 nm.

EXAMPLE 1h

Applications of the Pseudocercospora Immunoassay

Figures 2a and 2b illustrate the sensitivity of the Pseudocercospora Immunoassay for detecting the presence of P. herpotrichoides infection before the appearance of symptoms in both greenhouse- (Figure 2a) and field- (Figure 2b) infected wheat stems. Samples were prepared according to Example 1b.

Figures 3 and 4 illustrate the specificity of the Pseudocercospora Immunoassay for P. herpotrichoides when monospecific RAP Ig is used to coat the surface of microtiter plates. Wells were coated with unabsorbed (Figure 3) or monospecific (Figure 4) RAP Ig and reacted with fungal extracts described in Example 1a of S. tritici, F. roseum, P. herpotrichoides, and G. graminis diluted 1:100 in TBS/Tween.

Figure 5 shows that the assay is sensitive enough to detect picogram quantities of *P. herpotrichoides* antigen. The concentration of *P. herpotrichoides* antigen was determined by dialyzing a wheat strain fungal extract, against PBS as in Example 1b, evaporating a 0.5 ml sample to dryness at 37°, determining the weight of the dried residue and calculating the weight of the *P. herpotrichoides* antigen by subtracting out the weight of the salt.

EXAMPLE 1i

Microtiter Plate Pseudocercospora Immunoassay

The general assay protocol, following procedures described in Example 1g, consisted of 100 µl reagent additions per well, 30 min reagent incubations at 25° followed by three washes with 10 mM Tris, pH 7.4, 140 mM sodium chloride, 0.04% chloroacetamide, and 0.05% Tween 20 (TBSC/Tween). Monospecific RAP Ig was diluted to approximately 10 µg/ml in 0.1 M bicarbonate coating buffer at pH 9.6 and added to microtiter plate wells. Samples were diluted in TBSC/Tween and incubated with antibody coated wells. After the plates were incubated and washed, biotin-conjugated RAP Ig diluted to approximately 5 µg/ml in TBSC/Tween was applied to the wells. Final reagent additions consisted of streptavidin-alkaline phosphatase conjugate diluted to approximately 1 µg/ml in TBSC/Tween followed by p-nitrophenyl phosphate substrate solution (Kirkegaard and Perry Laboratories, Inc.). Color development in the wells was measured spectrophotometrically at 405 nm.

EXAMPLE 1jMembrane Format Pseudocercospora Immunoassay

5 Membrane based immunoassays were performed in a
Fast-Chek™ device (E-Y Labs, Inc., San Mateo, CA).
The device consists of a porous nitrocellulose
membrane backed with several pieces of absorbant
10 paper encased within a plastic body. The surface of
the nitrocellulose membrane serves as the solid
support for immobilizing the capture antibody.
Reagents applied to the surface of the solid support
wick through the pores of the membrane into the
15 absorbant paper backing. Reagents and buffers were
identical to those described in Example 1i except
where noted. One μ l of a 0.94 mg/ml solution of
monospecific RAP Ig was applied to the nitrocellulose
membrane. After drying the membrane for 5 min, 100
20 μ l of blocking solution (Buffer C, E-Y Labs, Inc.)
was applied and allowed to wick completely through
the membrane. 100 μ l of each additional reagent and
wash buffer (as described in Example 1i) were applied
sequentially and allowed to completely wick through
25 the membrane before the next reagent was applied.
The alkaline phosphatase substrate
5-bromo-4-chloro-3-indolyl phosphate, p-toluidine
salt was used in conjunction with nitroblue
tetrazolium chloride (BCIP/NBT Phosphatase Substrate,
Kirkegaard and Perry Labs, Inc.) to yield an
30 insoluble blue precipitate on the membrane.

EXAMPLE 2

Application of Tissue Grinder to

Pseudocercospora Immunoassay for

35 Extraction of Fungal Antigens from Plant Tissue

Fungal antigens were extracted from plant
tissue as described in Example 1b except that field

5 collected plant material was macerated into 5 ml of
extraction buffer using the tissue grinding apparatus
described in Figures 7 through 13.

The assay was performed in accordance with the
Examples 1a, and 1c through 1h.

10 Color development in the microtiter plate wells
was measured spectrophotometrically at 410 nm as
shown in Figure 6.

15 It will be apparent that the instant
specification and examples are set forth by way of
illustration and not limitation, and that various
modifications and changes may be made without
departing from the spirit and scope of the present
invention.

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CLAIMS

What is claimed is:

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1. A grinding device, comprising:

a cylindrical tube, one end of the tube
being closed with a circular base, the base having on
its interior face a plurality of radiating ridges
constituting a first grinding surface; and

10

a plunger having a first end and a second
end, the first end being cylindrical and having
radiating ridges complementary to the ridges of the
first grinding surface constituting a second grinding
surface, the first end of the plunger slidably
insertable into the cylindrical tube and axially
rotatable therein, the second end of the plunger
having a means for rotating the plunger within the
cylindrical tube to grind material positioned between
the first and second grinding surfaces, the space
between the inner surface of the tube and the first
end of the plunger such that ground material escapes
from the first and second grinding surfaces along the
inner surface of the tube but unground material does
not escape from the first and second grinding
surfaces.

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2. The grinding device of Claim 1 having in
addition a grind enhancing and alignment means, the
grind enhancing and alignment means comprising:

30

a peg protruding concentrically along the
tube's longitudinal axis from the interior face of
the base; and

35

a cavity recessed concentrically along
the plunger's longitudinal axis from the first end of
the plunger, the cavity having dimensions such that

5 the plunger rotatably fits over the peg when the first grinding surface contacts the second grinding surface.

10 3. The grind enhancing and alignment means of Claim 2, wherein the space between the peg and the inner wall of the cavity is less than the space between the inner wall of the tube and the outer surface of the plunger head.

15 4. The grinding device of Claim 1 wherein the circular base is perpendicular to the longitudinal axis of the tube.

20 5. The grinding device of Claim 1 wherein the first and second grinding surfaces are at an angle of 15°-25° from the horizontal.

6. The grinding device of Claim 1 wherein the second end of the plunger has a close, rotating fit within the tube.

25 7. A method for assaying the fungus of the genus Pseudocercospora, comprising:

- 30 A. extracting fungal antigens from plant tissue into an aqueous medium;
- B. forming a reaction system on a solid support coated with a high titer polyclonal capture antibody that is reactive with the fungus Pseudocercospora, and contacting said surface with :
- 35 1. the aqueous medium containing the fungal antigen;

- 5 2. then adding a polyclonal antibody that
 reacts with the antigen;
 3. then adding a reporter mechanism;
 C. Measuring the signal from the reporter
 mechanism; and
10 D. Relating the signal to the presence or
 concentration of fungal antigen
 initially present in the aqueous medium.

8. The method of Claim 7 wherein the plant
tissue is obtained from plants susceptible to
15 infection by a member of the genus
Pseudocercospora.

9. The method of Claim 7 wherein the fungal
antigen is selected from the group consisting of a
20 Pseudocercospora herpotrichoides spore, hypha,
cytoplasmic fraction, toxin, enzyme, exoantigen,
elicitor, polysaccharide, protein, nucleic acid, or
any aggregate or regularly ordered structure
comprised of multiple or repeating units or
25 combinations of such structures or substances.

10. The method of Claim 7 wherein the fungal
antigen is a Pseudocercospora polysaccharide.

30 11. The method of Claim 7 wherein the aqueous
medium preferably comprises a phosphate buffered
saline solution, azide, and polyoxyethylenesorbatan
monolaurate.

35 12. The method of Claim 7 wherein the solid
support is selected from the group consisting of
microtiter plates, tubes, beads, magnetic particles,
and membranes.

13. The method of Claim 7 wherein the solid support is a polystyrene microtiter plate.

5 14. The method of Claim 7 wherein the high titer polyclonal capture antibody reagent is monospecific for the genus Pseudocercospora.

10 15. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a substance selected selected from the group consisting of enzymes, radioisotopes, chromophoric particles and fluorescent chromophores.

15 16. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a biotin-avidin-enzyme unit.

20 17. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with alkaline phosphatase.

25 18. The method of Claim 7 wherein the reporter mechanism comprises a biotin-avidin-enzyme conjugate coupled to the reactive polyclonal antibody and a substrate specific to the enzyme, the reporter mechanism indicating a color signal in the presence of Pseudocercospora antigen.

30

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19. The method of Claim 18 wherein the enzyme
is alkaline phosphatase.

20. The method of Claim 18 wherein the enzyme
substrate is p-nitrophenyl phosphate.

21. A method for assaying the fungus
Pseudocercospora herpotrichoides, comprising:

- A. extracting a Pseudocercospora
antigen from plant tissue into a
solution of Tris-buffered saline,
chloroacetamide, and polyoxyethylene-
sorbitan monolaurate;
- B. forming a reaction system on
polystyrene microtiter plate coated
with a high-titer polyclonal capture
antibody monospecific for the
Pseudocercospora antigen and
contacting said microtiter plate with:
 1. the solution containing the fungal
antigen;
 2. then adding a polyclonal antibody-
biotin conjugate that reacts to
the Pseudocercospora antigen;
 3. then adding a reporter mechanism
including a streptavidin-alkaline
phosphatase conjugate and
p-nitrophenyl phosphate,
in proportions such that a signal from
the reporter mechanism is related to
the presence of or concentration of
Pseudocercospora antigen initially
present in the aqueous medium;

C. Measuring the signal from the reporter mechanism; and

D. Relating the signal to the presence or concentration of Pseudocercosporella antigen initially present in the aqueous medium.

22. A method for assaying the fungus Pseudocercosporella herpotrichoides, comprising:

A. extracting a Pseudocercosporella antigen from plant tissue into a solution of Tris-buffered saline, chloroacetamide, and polyoxyethylene-sorbitan monolaurate;

B. forming a reaction system on a membrane coated with a high-titer polyclonal capture antibody monospecific for the Pseudocercosporella antigen and contacting said membrane with:

1. the solution containing the fungal antigen;
2. then adding a polyclonal antibody-biotin conjugate that reacts to the Pseudocercosporella antigen;
3. then adding a reporter mechanism including a streptavidin-alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl phosphate substrate,

in proportions such that a signal from the reporter mechanism is related to the presence of or concentration of Pseudocercosporella antigen initially present in the aqueous medium;

5 C. Measuring the signal from the reporter mechanism; and

D. Relating the signal to the presence or concentration of Pseudocercospora antigen initially present in the aqueous medium.

10 23. The method of Claim 7 wherein the aqueous medium preferably comprises a Tris-buffered saline solution, chloroacetamide and polyoxyethylenesorbitan monolaurate.

15 24. The method of Claim 7 wherein the solid support is a membrane.

20 25. The method of Claim 7 wherein the reactive polyclonal antibody is labelled with a biotin-streptavidin-enzyme unit.

25 26. The method of Claim 7 wherein the reporter mechanism comprises a biotin-streptavidin-enzyme conjugate coupled to a reactive polyclonal antibody and a substrate specific to the enzyme, the reporter mechanism indicating a color signal in the presence of Pseudocercospora antigen.

30 27. The method of Claim 26 wherein the enzyme is alkaline phosphatase.

35 28. The method of Claim 26 wherein the enzyme substrate is 5-bromo-4-chloro-3-indolyl phosphate.

29. A kit to test for the presence of
5 Pseudocercospora antigen in cereal crop,
comprising:
a tissue grinder for the extraction of
Pseudocercospora antigen from plant tissue;
antigen extraction buffer in which to
10 place the antigen;
a solid support for the attachment of an
immunoassay;
a preparation of labelled antibody which
includes an enzyme and an antibody reactive with the
15 Pseudocercospora antigens;
a preparation of antibody monospecific
for Pseudocercospora antigens; and
a preparation of enzyme substrate which
is specific for the enzyme of the labelled antibody
20 preparation and indicates this specificity by a color
change.

30. A kit of Claim 29 wherein:
the antigen extraction buffer is
25 Tris-buffered saline, chloroacetamide, and
polyoxyethylene-sorbitan monolaurate;
the solid support is a polystyrene
microtiter plate;
the labelled antibody is a system
30 comprising a polyclonal antibody-biotin conjugate
that reacts to the Pseudocercospora antigen and a
streptavidin-alkaline phosphatase conjugate reporter
mechanism; and
the enzyme substrate is p-nitrophenyl
35 phosphate.

31. A kit of Claim 29 wherein:

5 the antigen extraction buffer is
Tris-buffered saline, chloroacetamide, and
polyoxyethylene-sorbitan monolaurate;
the solid support is a membrane;
the labelled antibody is a system
10 comprising a polyclonal antibody-biotin conjugate
that reacts to the Pseudocercosporella antigen and a
streptavidin-alkaline phosphatase conjugate reporter
mechanism; and
the enzyme substrate is 5-bromo-4-
15 chloro-3-indolyl phosphate.

20

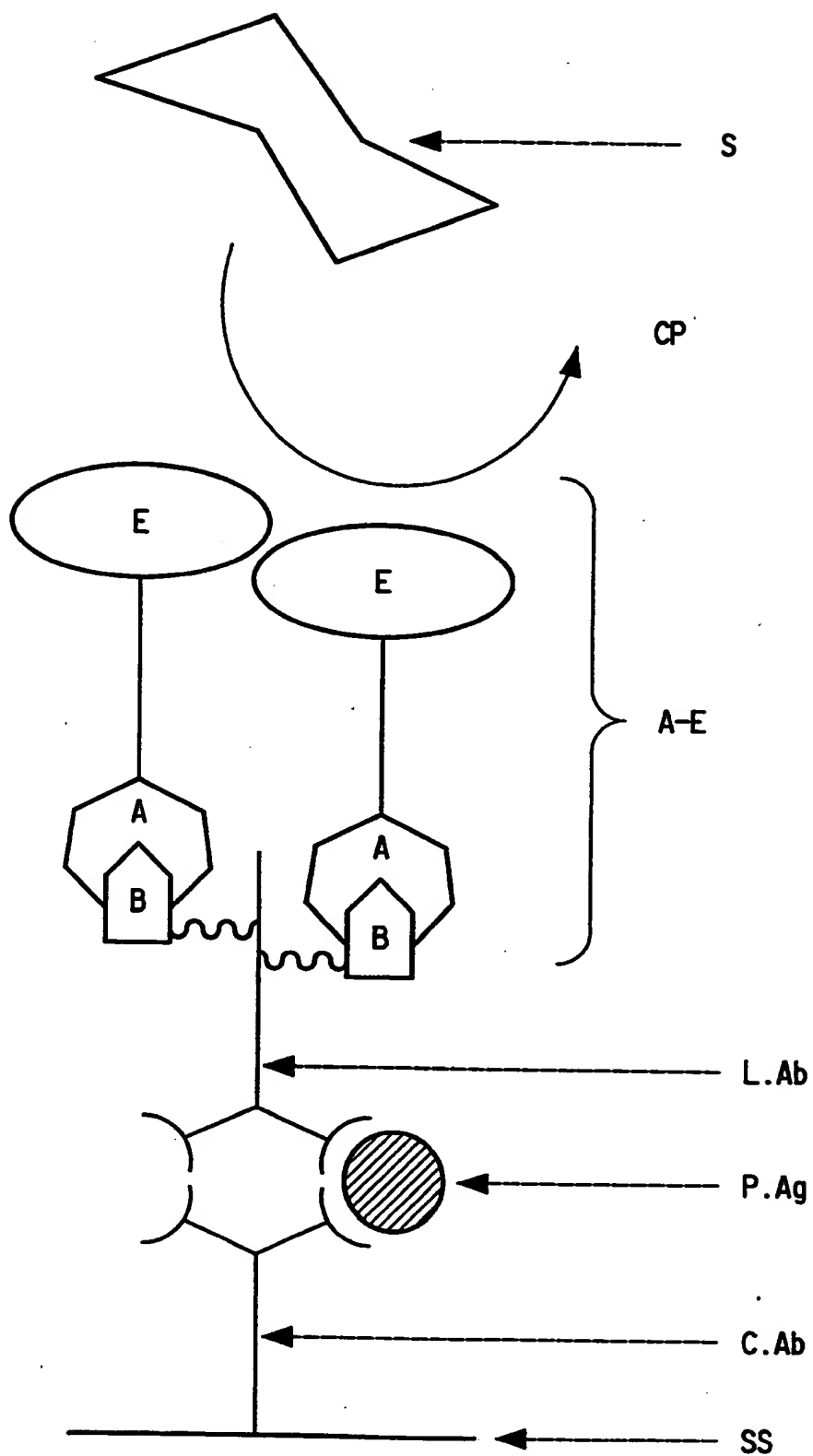
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FIG. 1

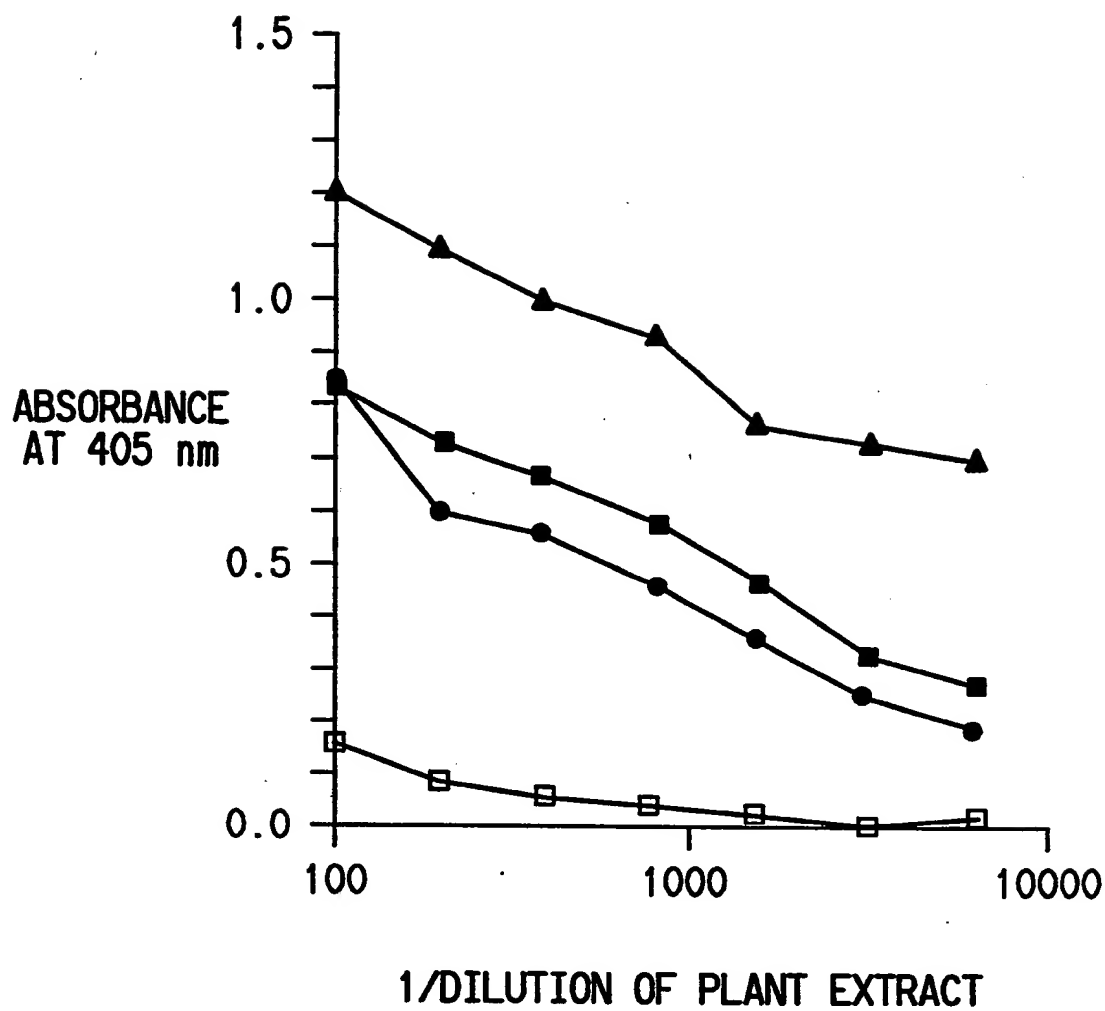
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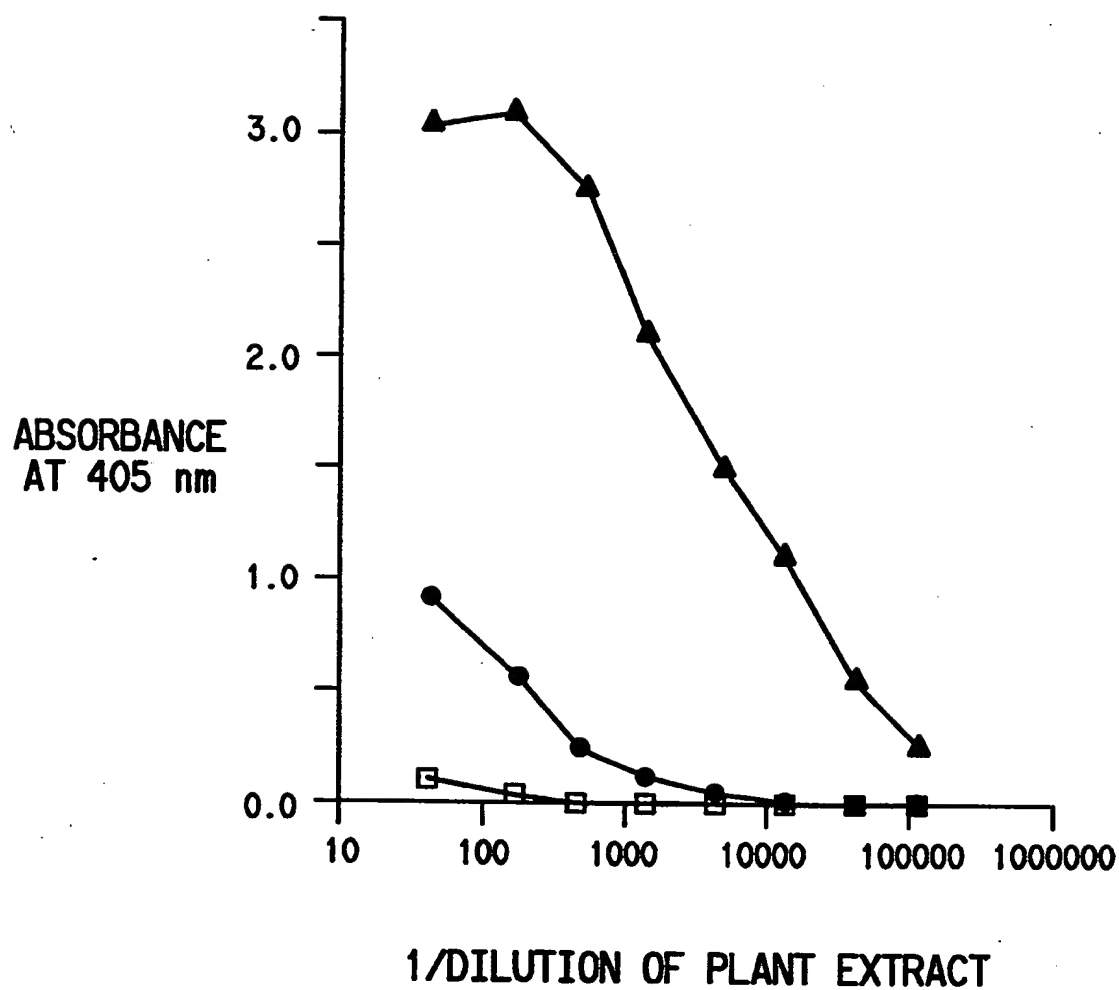
FIG. 2A



- UNINFECTED PLANT
- PRESYMPTOMATIC EYESPOT
- MODERATE EYESPOT
- ▲— SEVERE EYESPOT

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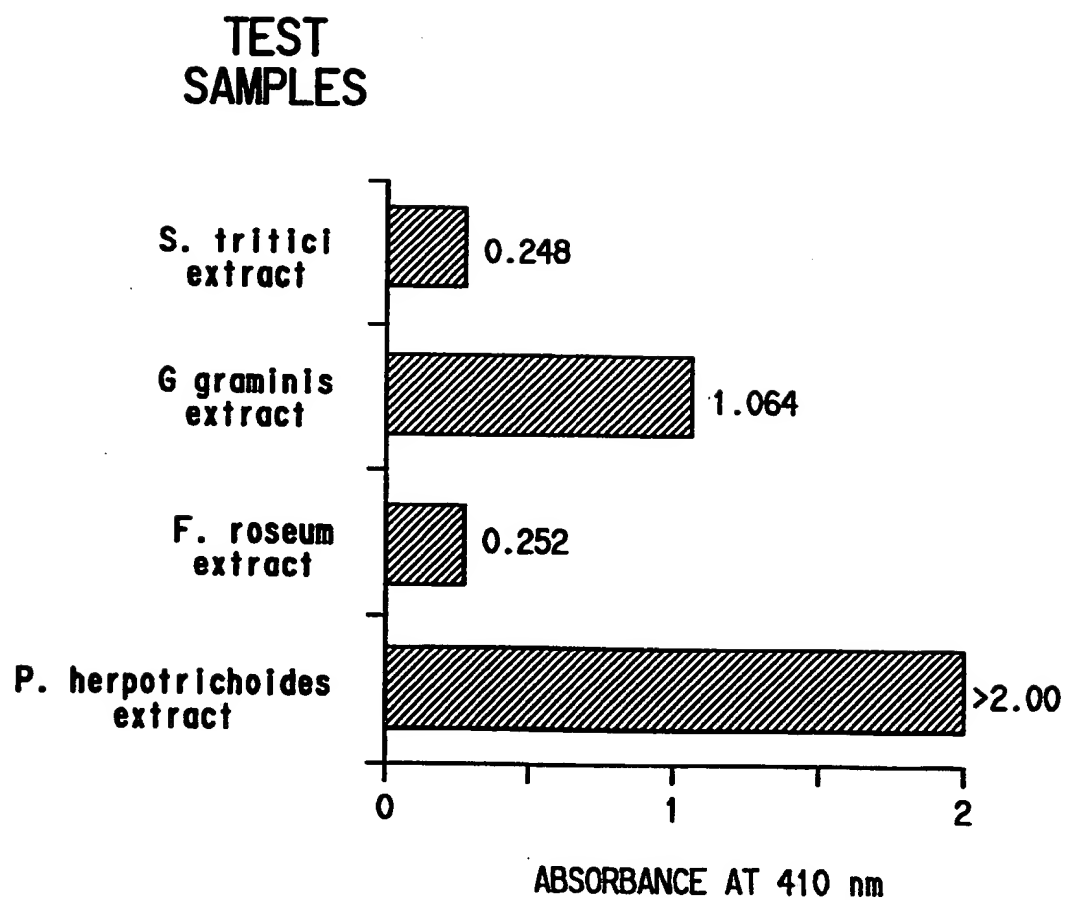
FIG. 2B



- UNINFECTED PLANT
—●— PRESYMPTOMATIC EYESPOT
—▲— SEVERE EYESPOT

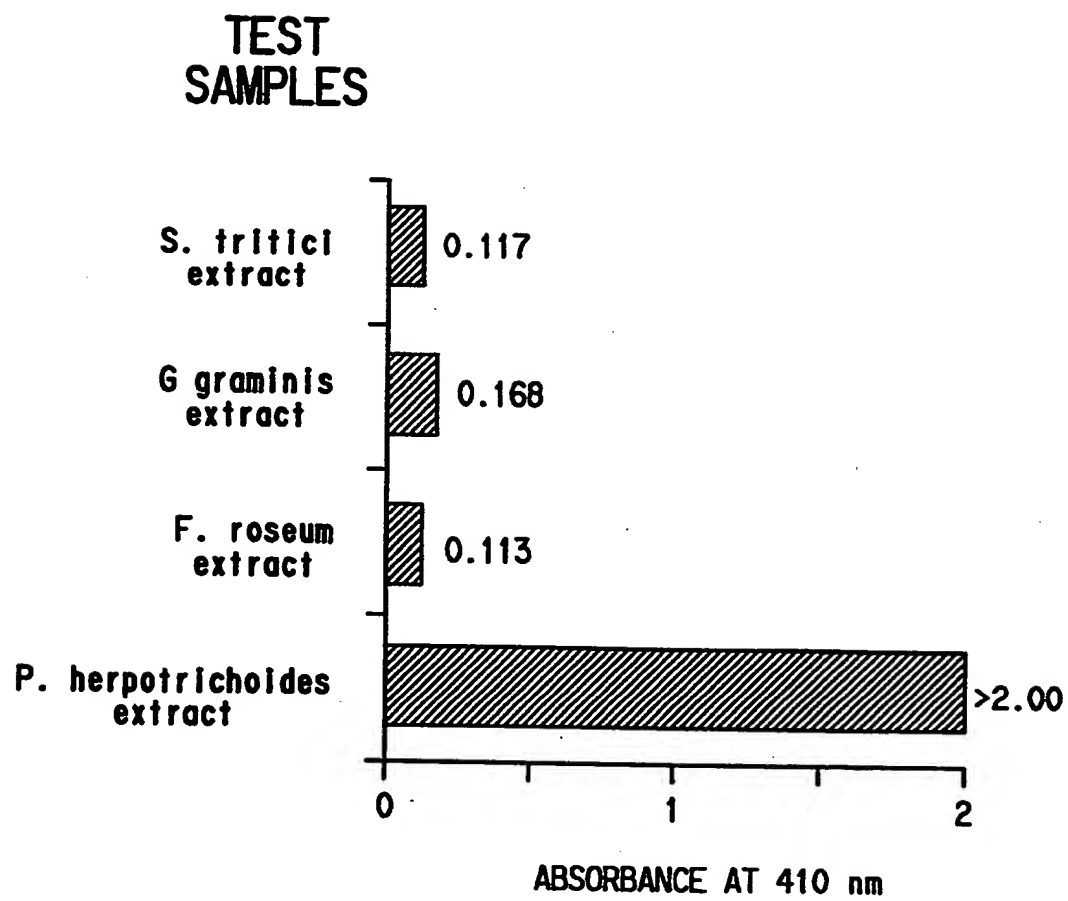
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FIG. 3



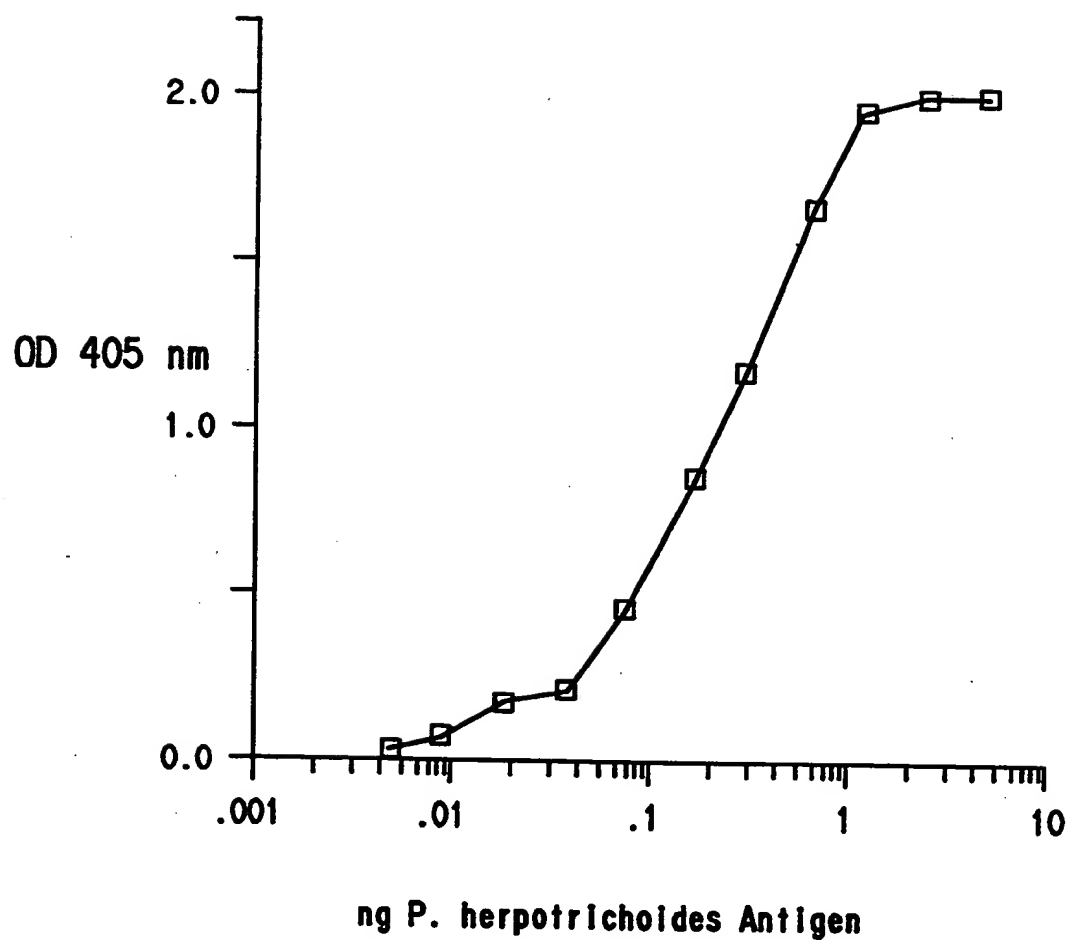
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FIG. 4



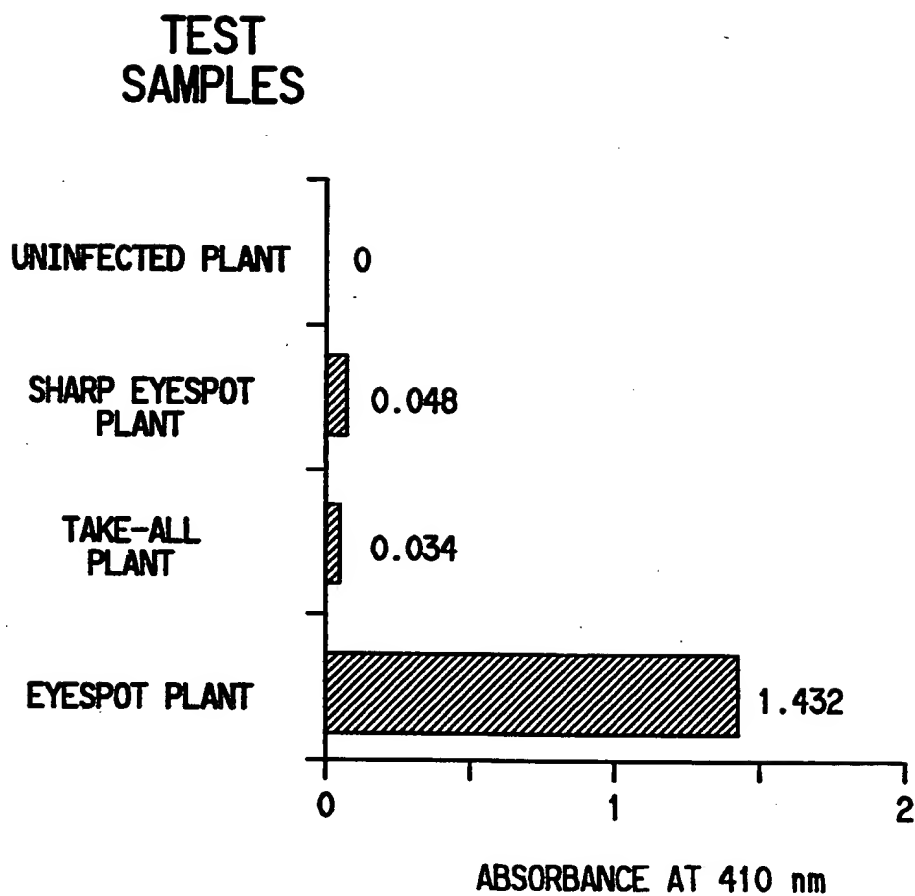
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FIG. 5



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FIG. 6



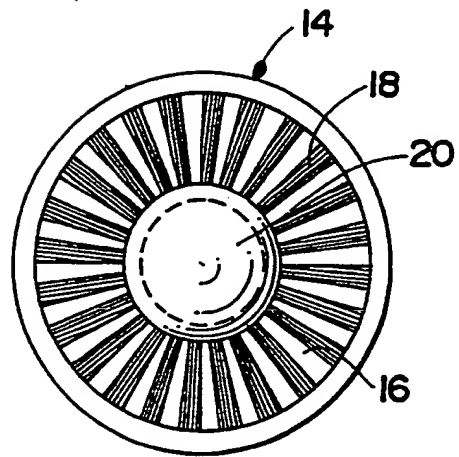
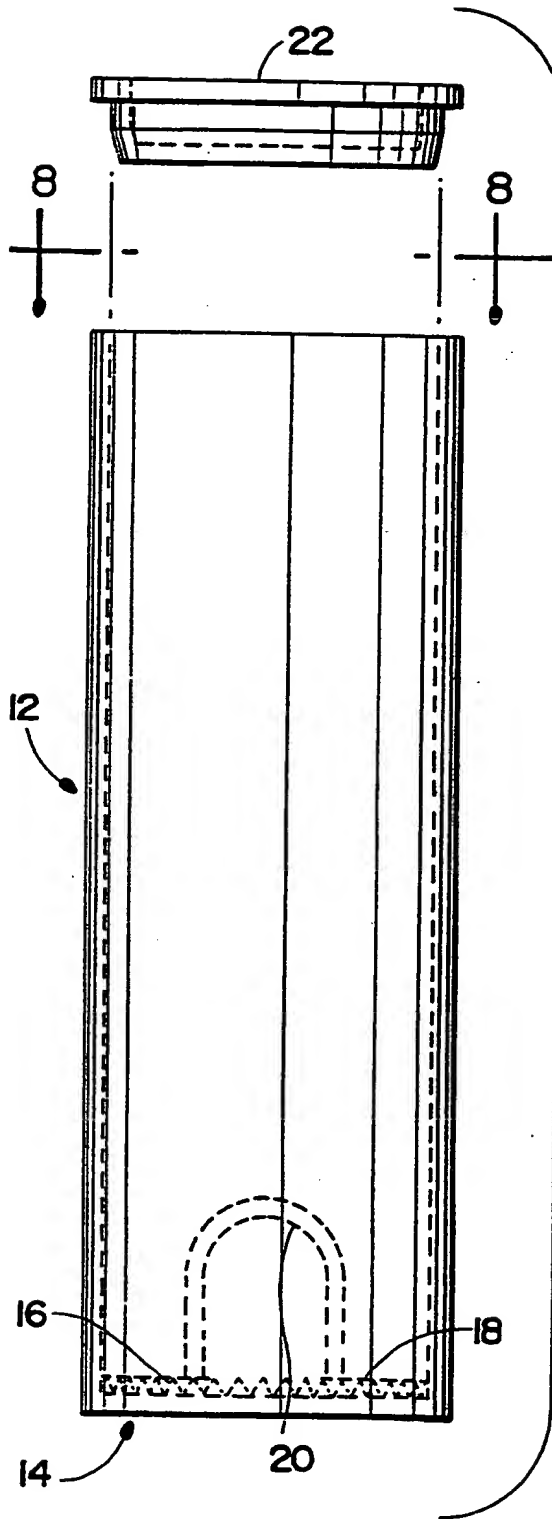


FIG. 8

FIG. 7

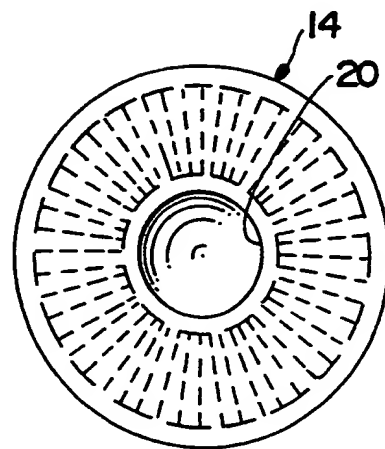


FIG. 9

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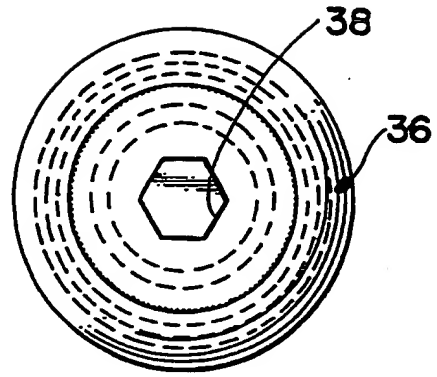
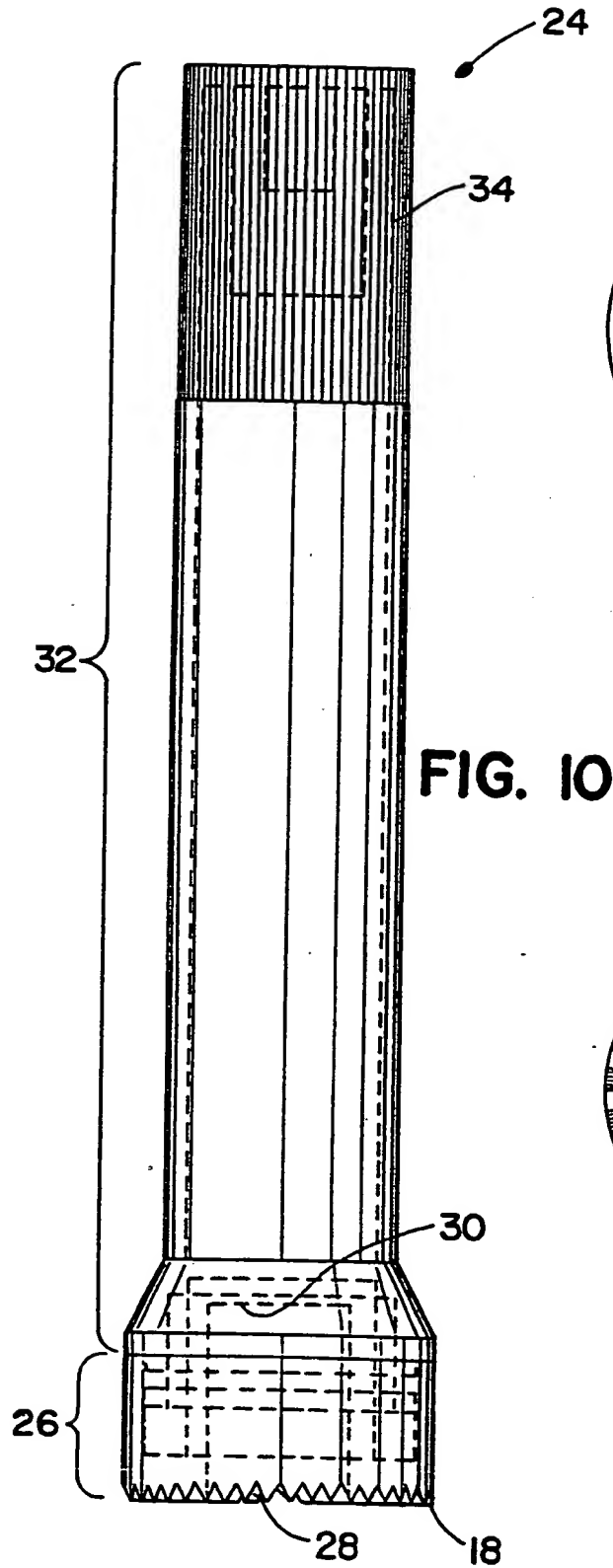


FIG. 11

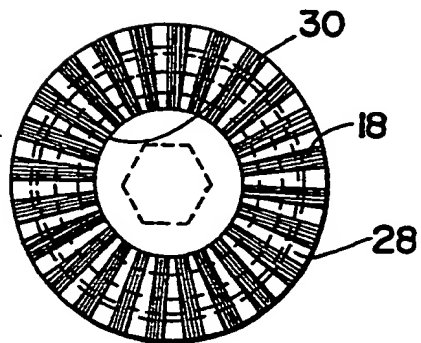
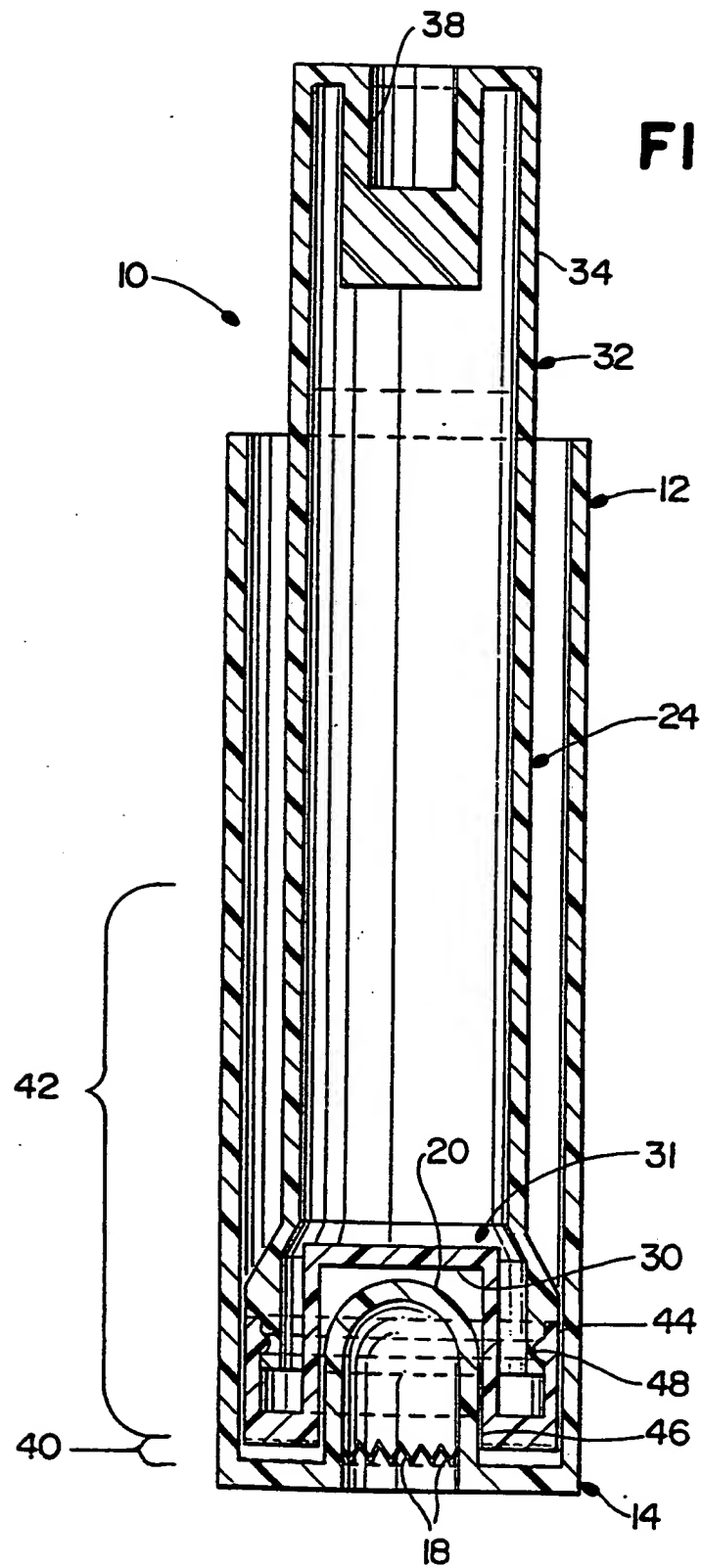


FIG. 12

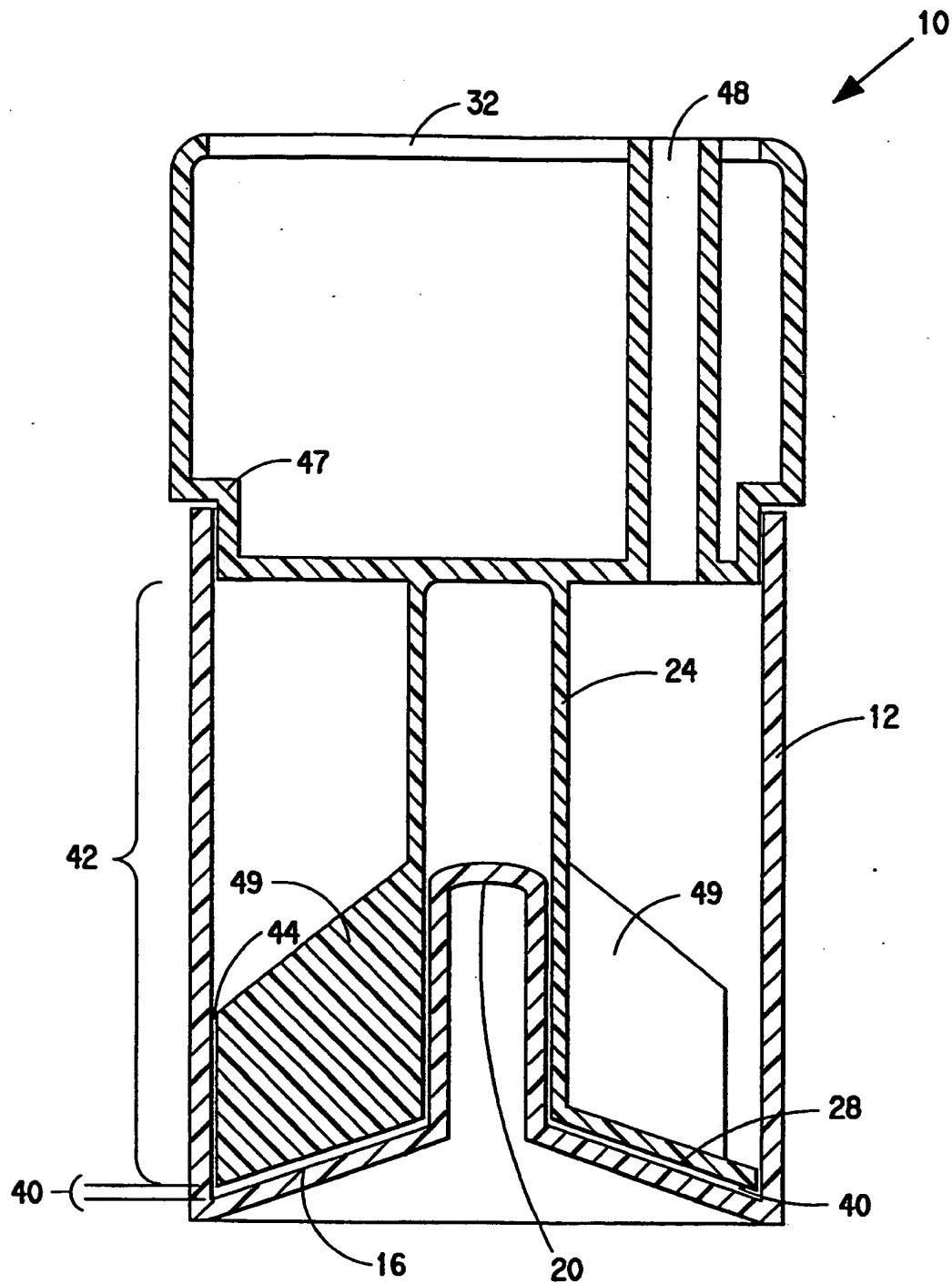
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FIG. 14

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